TRANSLATION FROM HUNGARIAN

[Handwritten notation:]
P99 2670
HU P99 02670A

[Ink stamp:]
COPY FOR PUBLICATION

66.510/SZE

ABSTRACT

Use of pharmaceutical composition containing appetite-suppressing peptide

The invention relates to a pharmaceutical composition containing an appetite-suppressing peptide or a fraction comprising an appetite-suppressing peptide, and to a method of appetite regulation by means of said peptide.

The invention further relates to the use of a pharmaceutical composition containing a peptide with the following amino acid sequence for the prevention or treatment of diseases or irregularities associated with impaired appetite regulation:

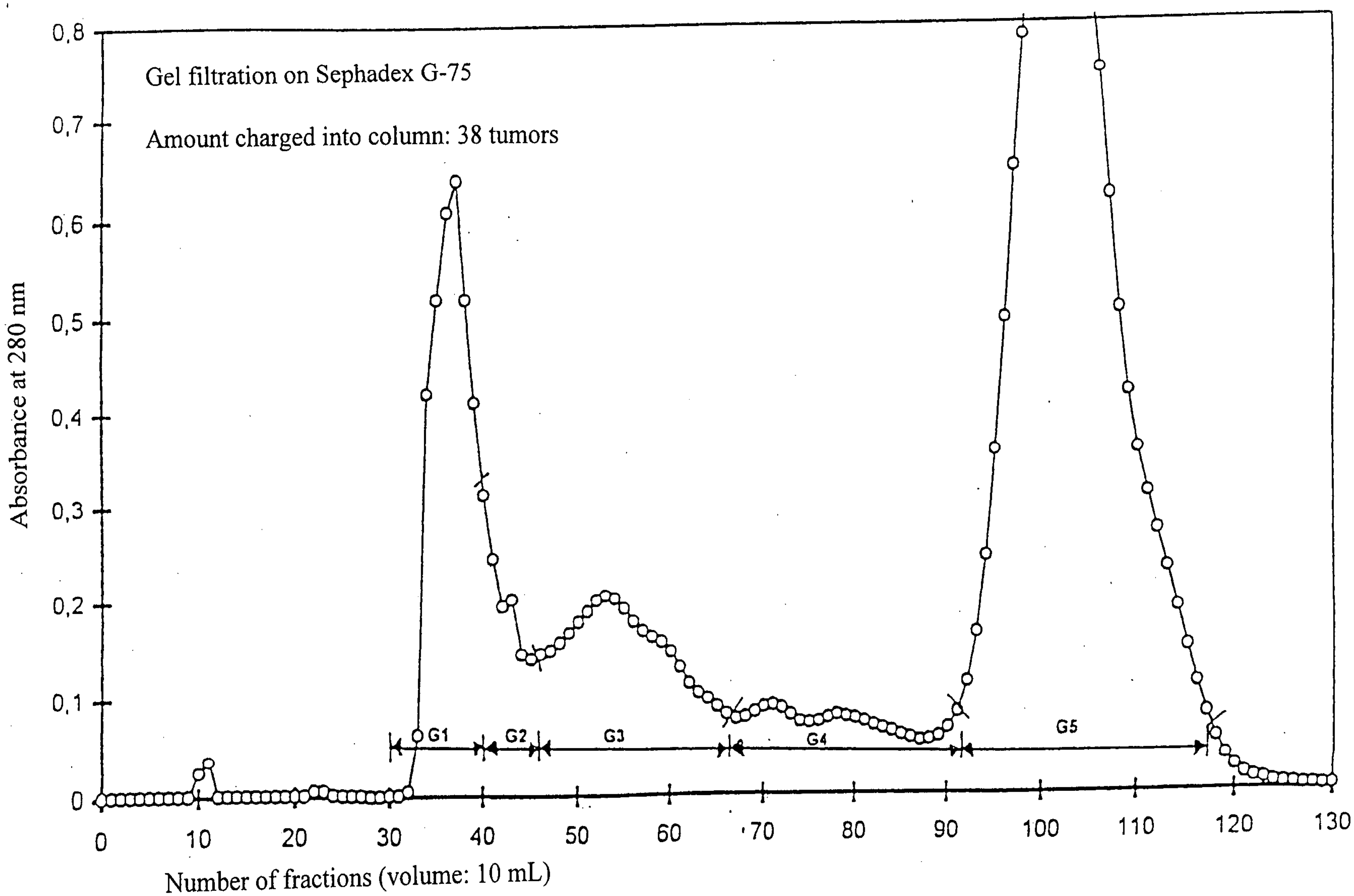
X^1 H X^2 D G S F S D E M N T X^3 L D X^4 L A X^5 X^6 D F I N W L X^7 X^8 T K I T D X^9

where X^1 is NH_2, DFPEEVAIVEELGRR, DFPEEVTIVEELGRR, DFPEEVNIVEELRRR, or a fragment thereof,
X^2 is Ala or Gly,
X^3 is Ile or Val,
X^4 is Asn, Ser or His,
X^5 is Ala or Thr,
X^6 is Arg or Lys,
X^7 is Ile or Leu,
X^8 Gln or His, and
X^9 is OH, Lys, Arg, Arg-Lys, Lys-Arg, Arg-Arg or Lys-Lys.

[Handwritten notation:] No illustrative figure. [Illegible signature]

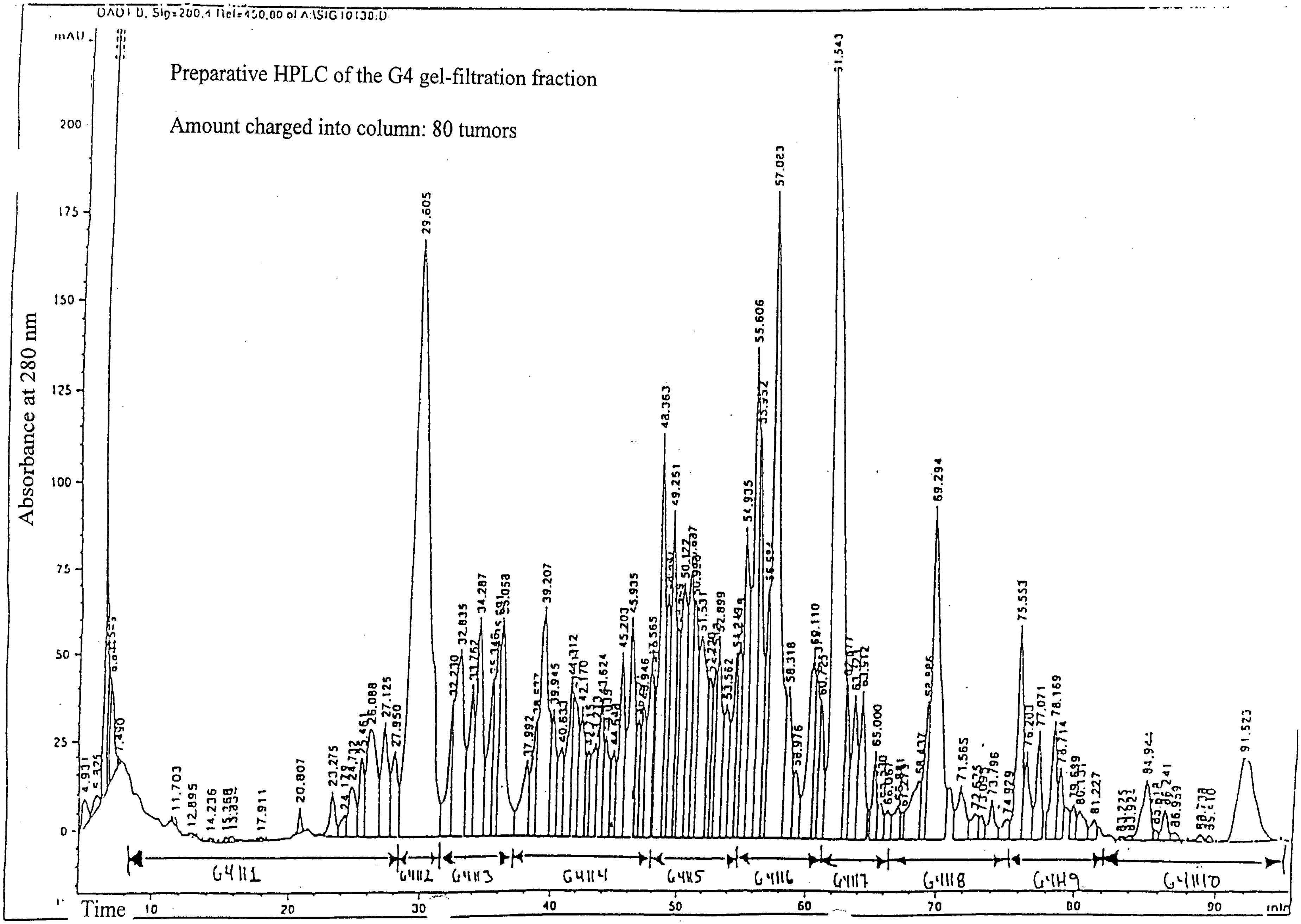
Fig. 1

[stamp:] COPY FOR PUBLICATION



[stamp:] COPY FOR PUBLICATION

Fig. 2



[stamp:]
S.B.G. & K.
International Patent Bureau
H-1062 Budapest, Andrássy út 113
Tel.: 34-24-950; Fax: 34-24-323

66.510/SZE

[stamp:]
COPY FOR PUBLICATION

Use of pharmaceutical composition containing appetite-suppressing peptide

The invention relates to a pharmaceutical composition containing an appetite-suppressing peptide or a fraction containing an appetite-suppressing peptide, and to a method of appetite regulation by means of said peptide.

Glucagon is produced by the pancreatic A-cell, and is released in response to low blood sugar levels. The main site of its action is the liver, where it stimulates glucose production. Hence in blood sugar homeostasis it is the main insulin-neutralizing hormone (Unger, R. H. and L. Orci, Glucagon, Diabetes Mellitus; 4th edition, New York, Elsevier, 104-120, 1990).

Glucagon is formed by limited proteolysis from a relatively large precursor. On the basis of molecular cloning of the glucagon gene it was found that the proglucagon precursor encodes not only glucagon but also two further glucagon-like peptides, named GLP-1 and GLP-2. GLP-1 and GLP-2 are encoded by separate exons, on the basis of which it can be assumed that their biological activity is different. Subsequently it was demonstrated that the proglucagon precursor grows through a different processing course in the three different tissues which are known to produce proglucagon: the pancreatic A-cell, the intestinal L-cell, and the central nervous system (CNS). Hence in the islet A-cell glucagon is excised selectively from the precursor, whereas GLP-1 and GLP-2 are selectively released from the intestinal L-cells and from the CNS (description: Unger, R. H. and L. Orci, Glucagon, Diabetes Mellitus, 4th edition, New York, Elsevier, 104-120, 1990).

Specific GLP-1 receptors have been identified (Thorens, B., Proc. Natl. Acad. Sci. USA 89, 8641-8645, 1992) which clearly differ from the glucagon receptor (L. J. Jelinek et al., Science 259, 1614-1616, 1993), and their tissue distribution is different (R. V. Campos et al., Endocrinology 134, 2156-2164, 1994); GLP-1 is released from L cells after meals, and acts as an incretin hormone (i.e., it promotes the glucose-induced release of insulin from the pancreatic B-

cells). Hence the GLP-1 receptor is expressed in large quantities on the surface of the islet B-cells (Moens et al., Diabetes, 45, 257-261, 1996).

It has been demonstrated that GLP-2 induces epithelial proliferation (Drucker, D. J. et al., Proc. Natl. Acad. Sci. USA 93, 7911-7916, 1996) and the treatment of gastrointestinal diseases by cells grown in GLP-2 nutrient medium has been described (Drucker, D. J. and Keneford, J. R., International Patent WO 96/32414). So far, GLP-2 receptors have not been reported.

Peptides originating from proglucagon, and feeding behavior

We have already reported the derivation and establishment of transplantable anorectic glucagonomas (O. D. Madsen at al., Endocrinology 133, 2022-2030, 1993) and of hypoglycemic insulinomas in the rat (O. D. Madsen et al., Proc. Natl. Acad. Sci. USA 85, 6652-6656, 1988). Such tumors can be derived from pluripotent MSL cells of common clonal origin (O. D. Madsen et al., J. Cell Biol. 103, 2025-2034, (1986), which show the maturation process directed toward the A-cell or B-cells, respectively (O. D. Madsen et al., Endocrinology 133, 2022-2030, 1993).

Glucagonoma associated with anorexia is very severe; it is of acute onset, and after a few days leads to a complete cessation of food intake. Such a severe anorexia is difficult to compare to other experimental tumors in rodents, and it can be assumed that glucagonoma produces a very strong satiety factor, which acts upon peripheral administration. It has already been shown earlier that the anorectic glucagonomas exhibit an unphysiological transformation which results in the formation of both glucagon and GLP-1 (O. D. Madsen et al., Endocrinology 133, 2022-2030, 1993). In fact, a nonanorectic glucagonoma variant was unable to process the precursor (O. D. Madsen et al., Scand. J. Clin. Lab. Invest. 55, Suppl. 20, 27-36, 1995). Mass loss is cited as one of the components also of the human glucagonoma syndrome (J. J. Holst, Glucagon-producing tumors; hormone-producing tumors of the gastrointestinal tract, New York, Churchill Livingstone, 57-84, 1985), although there is great variability among different patients (S. J. Bhathena et al., Glucagonoma and glucagonoma syndrome, Glucagon Physiology, Pathophysiology and Morphology of the Pancreatic A-cells, New York, Elsevier, 413-438, (1981).

Glucagon

Glucagon has been shown to participate in the regulation of the spontaneous intake of food in rats, but its general effect is minimal, and it exerts its action on the liver by vagal connections (N. Geary et al., Am. J. Physiol. 264, R116-R122, 1993). This action can only be observed if the glucagon is administered by portal infusion, whereas drug doses administered intraperitoneally have no effect on the intake of food in fasted rats (O. D. Madsen et al., Endocrinology 133, 2022-2030, 1993).

GLP-1

The central role played by GLP-1 in nutritional regulation has been reported (M. D. Turton et al., Nature 379, 69-72, 1996). Intracerebroventricular (ICV) administration of GLP-1 inhibits food intake in fasted rats. Renewed peripheral administration of GLP-1 had no effect on feeding behavior (M. D. Turton et al., Nature 379, 69-72, 1996; O. D. Madsen et al., Endocrinology 133, 2022-2030), on the basis of which it may be assumed that GLP-1 produced by the tumor cannot significantly contribute to the observed anorexia.

We found that when peripherally administered, GLP-2 has a strong effect in inhibiting food intake.

It can be assumed that GLP-2, which in a normal case is released together with GLP-1 from intestinal L-cells, plays its own separate role as a satiety factor.

Accordingly, the invention relates to the use of a pharmaceutical composition which contains, together with a pharmaceutically acceptable auxiliary substance or vehicle, the HPLC fraction of a glucagonoma tumor extract prepared by acid ethanolic extraction, gel filtration and preparative HPLC, said fraction being shown in Fig. 2 as fraction G4H9, and containing, as its main component (more than 40%) a glucagon-like peptide 2 (GLP-2), or one component of said fraction, or two or more components of said fraction.

A further object of the invention is the use of a pharmaceutical composition containing glucagon-like peptide-2 (GLP-2) or a variant or homolog thereof for the prevention or treatment of diseases or irregularities associated with impaired appetite regulation.

A further object of the invention is the use of a pharmaceutical composition containing a peptide with the following amino acid sequence:

X^1 H X^2 D G S F S D E M N T X^3 L D X^4 L A X^5 X^6 D F I N W L X^7 X^8 T K I T D X^9

where X^1 is NH_2, DFPEEVAIVEELGRR, DFPEEVTIVEELGRR, DFPEEVNIVEELRRR, or a fragment thereof,

X^2 is Ala or Gly,

X^3 is Ile or Val,

X^4 is Asn, Ser or His,

X^5 is Ala or Thr,

X^6 is Arg or Lys,

X^7 is Ile or Leu,

X^8 I Gln or His, and

X^9 is OH, Lys, Arg, Arg-Lys, Lys-Arg, Arg-Arg or Lys-Lys.

for the prevention or treatment of diseases or irregularities associated with impaired appetite regulation.

A further object of the invention is a method of treating diseases or irregularities associated with impaired appetite regulation, said method consisting in administering to an individual needing such treatment an amount of the peptide defined herein that is sufficient for reducing the appetite or inducing satiety in said individual.

A further object of the invention is the use of the above-defined peptide for preparation of a medicinal drug for the treatment of diseases or irregularities associated with impaired appetite regulation.

In the present description, the expression "peptide" is understood to mean that it contains the GPL-2 peptide or a precursor form thereof, as well as its functional fragment which essentially possesses the activity of the full-length peptide. Furthermore, the expression "peptide" is intended to include the homologs of said peptide as well. Such homologs contain an amino acid sequence showing a degree of identity of at least 50%, e.g., 75%, most characteristically 90% identity with the amino acid sequence of human GLP-2. The degree of identity can be determined by conventional methods, cf., e.g., articles by Altshul et al. (Bull. Math. Bio. 48, 603-616, 1986), and Henikoff and Henikoff (Proc. Natl. Acad. Sci. USA 89, 10915-10919, 1992).

Homologs of the invention possess one or more amino acid substitutions, deletions or additions. These changes may be of a minor extent, i.e., conservative amino-acid substitutions which do not have a significant effect on the foldability or activity of the peptide; small deletions, characteristically of a magnitude of from one to about five amino acids; small amino- and carboxy-terminal extensions such as, e.g., an amino-terminal methionine residue; a small, linker peptide consisting a maximum of about 15 amino acid residues, or a small extension which makes purification possible, such as a polyhistidine part, an antigenic epitope or a binding domain. For a general discussion, see the article of Ford et al. (Protein Expression and Purification 2, 95-107, 1991). Conservative substitutions are substitutions within the group of basic amino acids (such as arginine, lysine, and histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, and valine), aromatic amino acids (such as phenylalanine, tryptophane, and tyrosine), and small amino acids (such as glycine, alanine, serine, threonine, and methionine).

The homolog can be an allelic variant, i.e., an alternative gene form originating from a mutation, or an altered peptide encoded by a mutated gene which, however, possesses an essentially identical activity with native GLP-2 peptide. For this reason these mutations can be silent (there is no change in the encoded peptide) or may encode peptides having an altered amino acid sequence.

The peptide homolog can also be a species homolog, i.e., a peptide having a similar activity derived from another species. Examples of GPL-2 species homologs are human GLP-2, bovine GLP-2, rat GLP-2, hamster GLP-2, guinea-pig GLP-2 and porcine GLP-2.

In one advantageous embodiment of the invention the GLP-2 peptide is such that X^1 represents NH_2, X^2 is Ala, X^3 is Ile, X^4 is Asn, X^5 is Ala, X^6 is Arg, X^7 is Ile, X^8 is Gln and X^9 is OH. Specifically, the peptide has the following amino acids sequence:

H A D G S F S D E M N T I L D N L A A R D F I N W L I Q T K I T D (human GLP-2); or

H A D G S F S D E M N T I L D N L A T R D F I N W L I Q T K I T D (rat GLP-2); or

H A D G S F S D E M N T V L D N L A T R D F I N W L L H T K I T D (porcine GLP-2).

A homolog of the peptide may be isolated by preparing the genomic or cDNA library of a cell of the species in question, and isolating DNA sequences which code for the complete homolog or part thereof, by screening with the use of synthetic oligonucleotide probes, employing standard methods such as described by Sambrook et al. (Molecular Cloning: A laboratory manual; 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989) or by means of polymerase chain reaction (PCR) using specific primers, as described by Sambrook et al. (see above).

The invention also relates to a composition containing a variant of the GLP-2 peptide. The variant is a peptide in which one or more amino acid residues are substituted by other amino acid residues. In one particularly advantageous embodiment the Ala is replaced by Gly in the position 2 of the mature peptide. As may be expected, this variant will show a longer plasma half-life than the native peptide, which is of advantage, because the dose necessary for adequate appetite suppression or attainment of a satiety effect will generally be lower.

The GLP-2 peptide or its above-defined homolog or variant can be prepared by recombinant DNA techniques according to methods that have proved very satisfactory in the art.

To be more precise, the DNA sequence encoding the GLP-2 peptide can be isolated or synthesized on the basis of the published human preproglucagon DNA sequence (see J. W. White et al., Nucleic Acids Res. 14, 4719-4730 (1986); G. I. Bell et al., Nature 304, 368-371, 1983), for example, by preparing a genomic or cDNA library from an appropriate tissue and screening for DNA sequences coding for the full GLP-2 peptide or part thereof by hybridization using synthetic oligonucleotide probes in accordance with standard methods (see Sambrook et al. above). For this purpose the DNA sequence encoding the GLP-2 is preferably of human origin.

The DNA construct coding for the GLP-2 peptide may also be prepared by synthesis, using well-established methods, for example, by the phosphoamidite method described by Beaucage and Caruthers (Tetrahedron Letters 22, 1859-1869, 1981) or by the method described by Matthes et al. (EMBO Journal 3, 801-805, 1984). According to the phosphoamidite method, the oligonucleotides are synthesized, for example, by means of an automatic DNA synthesizer, then purified, annealed, ligated and cloned in appropriate vectors.

Furthermore, the DNA construct may be of mixed synthetic and genomic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin (as appropriate), which may be prepared

by ligation of fragments of synthetic, genomic or cDNA origin corresponding to various parts of the full DNA construct, using standard techniques.

The DNA construct can also be prepared by polymerase chain reaction using specific primers, for example, as described in U. S. Patent 4,683,202 or by Saiki et al. (Science 239, 487-491, 1988) or Sambrook et al. (see above).

In a currently preferred embodiment, the DNA construct contains the DNA sequence shown in Fig. 3 of the article by Bell et al. (Nature 304, 368-371, 1983), and also contains nucleic acid sequences coding for GLP-2 which, as a result of the degenerated state of the genetic code, differ from the DNA sequence shown in Fig. 3 of the article of Bell et al. (see above). Moreover, the DNA construct contains nucleic acid sequences which hybridize to a (genomic, synthetic, cDNA or RNA) nucleic acid molecule coding for human GLP-2 under very stringent conditions (i.e., presoaking in 5X SSC, prehybridizing for one hour at 40 °C in 20% formamide solution, 5X Dendhardt's solution, 50 mM sodium phosphate, at pH 6.8 with 50 μg of denatured ultrasound-treated calf thymus DNA, followed by hybridizing at about 40 °C in the same solution supplemented with 100 μg of ATP for 18 hours, then washing at about 45 °C with 0.4 X SSC). This could be, e.g., a DNA sequence coding for GLP-2 originating from another species, for example rat, bovine, hamster, guinea pig or porcine GLP-2.

For expression of GLP-2, the DNA construct coding for the GLP-2 peptide is inserted into an appropriate recombinant vector. This can be any vector conventionally used in recombinant DNA procedures, and the choice of vector often depends on the host cell into which the vector is to be introduced. Thus the vector can be an autonomously replicating vector, i.e., a vector present in an extrachromosomal form, whose replication is independent of chromosomal replication, e.g., a plasmid. As an alternative possibility, the vector can be one which, when introduced into a host cell, integrates into the genome of the host cell, and replicates together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence coding for the GLP-2 peptide links operably to further segments needed for DNA transcription. In general, the expression vector originates from a plasmid or viral DNA, or may contain elements of both. The expression "operably linked" means that the segments are arranged so that they function in

accordance with the intended purpose, e.g., the transcription begins with a promoter and continues via the DNA sequence which codes for the peptide.

The promoter may be any sequence which shows a transcriptional activity in the host cell chosen, and originates from genes coding for proteins that are homologous or heterologous to the host cell.

Suitable promoters for directing the transcription of the DNA coding for the GLP-2 peptide are, e.g., in mammalian cells, the SV40 promoter (Subramani et al., Mol. Cell Biol. 1, 854-864, 1981), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 809-814, 1983) or 2 major late promoters of the adenovirus.

A suitable promoter for use in insect cells is, e.g., the polyhedrin promoter (U.S. Patent 4,745,051); Vasuvedan et al., FEBS Lett. 311, 7-11, 1992), the P10 promoter (J. M. Vlak et al., J. Gen. Virology 69, 765-776, 1988), the Autografica californica polyhedrosis virus basic protein promoter (European Patent 397,485), the baculovirus immediate early gene-1 promoter (European Patent 397,485), or the baculovirus 39K delayed-early gene promoter (U. S. Patents 5,155,037 and 5,162,222).

Promoters suitable for use in yeast host cells are, e.g., the promoters originating from the glycolytic genes of yeast (Hitzeman et al., J. Biol. Chem. 255, 12073-12080, 1980); Alber and Kawasaki, J. Mol. Appl. Gen. 1. 419-434, 1982) or the promoters originating from alcohol dehydrogenase genes (Young et al., Genetic Engineering of Microorganisms for Chemicals (edited by Hollaender et al.), Plenum Press, New York (1982). or the TPI1 promoters (U. S. Patent 4,599,311) or ADH2-4c promoters (Russell et al., Nature 304, 652-654, 1983).

Promoters suitable for use in filamentous fungus host cells are, for example, the ADH3 promoter (McKnight et al., EMBO J. 4, 2093-2099, 1985) or the tpiA promoter. Other usable promoters are, e.g., the promoters originating from genes coding for *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic acid proteinase, *A. niger* neutral alpha-amylase, *A. niger* acid-stable alpha-amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. TAKA amylase and gluA promoters are preferred.

For use in bacterial host cells, suitable promoters include, e.g., the promoter of the *Bacillus stearothermophilus* maltogene amylase gene, *Bacillus licheniformis* alpha-amylase gene, *Bacillus amyloliquefaciens* BAN amylase gene, *Bacillus subtilis* alkaline protease gene, or *Bacillus pumilus* xyloxidase gene, or the lambda P_R or P_L promoters or *E. coli* lac, trp or tac promoters.

The DNA sequence coding for GLP-2 peptide may also be linked operably, if necessary, to a suitable terminator such as, e.g., human growth hormone terminator (Palmiter et al., op. cit.) or (in the case of fungus host cells) to TPI1 terminator (Alber and Kawasaki, op. cit.) or ADH3 terminator (McKnight et al., op. cit.). The vector may contain further elements as well, e.g., polyadenylation signals (such as those originating from the SV40 or adenovirus 5 Elb region), transcriptional enhancer sequences (e.g., the SV40 enhancer) and translational enhancer sequences (e.g., sequences encoding the adenovirus VA RNAs).

The recombinant vector may further contain a DNA sequence which makes possible the replication of the vector in the host cell in question. Such a sequence (when the host cell is a mammalian cell) is, e.g., the SV40 replication origin.

When the host cell is a yeast cell, suitable sequences which make possible the replication of the vector are the REP 1-3 replication genes and replication origin of the yeast 2μ plasmid.

The vector may also contain a selectable marker, for example a gene whose product complements a defect in the host cell, such as, e.g., the gene coding for dihydrofolate reductase (dhfr) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, Gene 40, 125-130, (1985), or a gene which confers resistance to a drug, e.g., to ampicillin, kanamycin, tetracycline, chloramphenicol, neomycin, hygromycin or methotrexate. In the case of filamentous fungi, selectable markers include amdS, pyrG, argB, niaD, and sC.

To direct the GLP-2 peptide in the direction of the host cells' secretion path, the recombinant vector may also be provided with a secretory signal sequence (also known as leader sequence, preprosequence or presequence). The secretory signal sequence is linked to the peptide-coding DNA sequence in the correct reading frame. The secretory signal sequences are generally linked to the 5' end of the peptide-coding DNA sequence. The secretory signal sequence may be of the

kind which, in a normal case, can also be associated with the gene or may originate from a gene that codes for another secreted protein.

For secretion from yeast cells the secretory signal sequence may code for any signal peptide that ensures effective direction of the expressed peptide toward the secretory path of the cell. The signal peptide may be a naturally occurring signal peptide or a functional part thereof, or a synthetic peptide. It has been found that a suitable signal peptide is the alpha-factor signal peptide (cf. U. S. Patent 4,870,008), the signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et al., Nature 289, 643-646, 1981), a modified carboxypeptidase signal peptide (cf. L. A. Walls et al., Cell 48, 887-897, 1987), the yeast BAR1 signal peptide (see International patent WO 87/02670) or the yeast aspartic acid protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 127-137, 1990).

In the interest of an effective secretion in the yeast, a leader-peptide-coding sequence can also be inserted downstream of the signal sequence and upstream of the DNA sequence coding for the GLP-2 peptide. The role of the leader peptide is to ensure directing the expressed peptide from the endoplasmic reticulum to the Golgi apparatus and, further, to a secretory vesicle, in order that it be secreted into the culture medium (i.e., that the peptide be exported through the cell wall or at least through the cell membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast's alpha-factor leader (whose use is described, e.g., in U. S. Patent 4,546,082, in European Patent 16,202, European Patent 123,294, European Patent 123,544 and European Patent 163,529). As an alternative possibility, the leader peptide can also be a synthetic leader peptide, i.e. a leader peptide that does not occur in nature. Synthetic leader peptides may be prepared, e.g., by the method described in International patents WO 89/02463 and WO 92/11378.

For use in filamentous fungi the signal peptide may conventionally originate from a gene of *Aspergillus* sp. amylase or glucoamylase, a gene coding for *Rhizomucor miehei* lipase or protease, or a gene coding for *Humicola lanuginosa* lipase. The signal peptide preferably originates from a gene coding for *A. oryzae* TAKA amylase, *A. niger* neutral alpha-amylase, *A. niger* acid-stable amylase or *A. niger* glucoamylase.

For use in insect cells, the signal peptide may conventionally originate from an insect gene (see International patent WO 90/05783), such as the signal peptide of the adipokinetic hormone precursor of the lepidoptera *Manduca sexta* (see U. S. Patent 5,023,328).

The methods used for ligating the GLP-2-coding DNA sequences, the promoter and optionally the terminator and/or secretory signal sequence and for inserting these into the appropriate vector containing the information required for replication are well known to persons skilled in the art (see, e.g. Sambrook et al., op. cit.). The DNA sequence coding for the GLP-2 peptide introduced into the host cell can be homologous or heterologous relative to the host cell in question. If it is homologous to the host cell, i.e., produced by the host cell in nature, it will characteristically be linked operably to a different promoter sequence or, if applicable, to a different secretory signal sequence and/or terminator sequence other than its natural environment. The term "homologous" is intended to include a cDNA sequence coding for a polypeptide native to the host organism in question. The term "heterologous" is intended to include a DNA sequence that is not expressed by the host cell in nature. Hence the DNA sequence can originate from another organism, or can be a synthetic sequence.

The host cell into which the DNA construct or recombinant vector according to the invention is introduced can be any cell capable of producing this peptide, and includes bacteria, yeasts, fungi and higher-order eukaryotic cells.

Bacterial host cells suitable for the production of GLP-2 peptide on cultivation are, for example, gram-positive bacteria such as *Bacillus* strains, e.g., *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus, B. megatherium or B. thuringiensis* strains or *Streptomyces* strains such as *S. lividans* or *S. murinus;* or gram-negative bacteria such as *Escherichia coli.* Transformation of the bacteria may be effected by protoplast transformation or by the use of competent cells in a manner known *per se* (Sambrook et al., see above).

When the peptide is expressed in bacteria, as for example in *E. coli*, the peptide may remain in the cytoplasm, characteristically in the form of insoluble granules (known as inclusion bodies), or it may be directed into the periplasmatic space with the aid of a bacterial secretory sequence. In the former case the cells are lysed and the granules are recovered and denatured, after which the peptide is refolded by diluting the denaturing agent. In the latter case the peptide may be

obtained from the periplasmic space by disrupting the cells, which may be accomplished, e.g., by ultrasound treatment or osmotic shock with the objective of releasing the contents of the periplasmic space and recovering the peptide.

Suitable mammary cell lines are, e.g., the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10), CHL (ATCC CCL39) or CHO (ATCC CCL 61) cell lines. Methods of transfecting the mammary cells and expressing the DNA sequences introduced into the cells are described, e.g., by Kaufman and Sharp, J. Mol. Biol. 159, 601-621, 1982; Southern and Berg, J. Mol. Appl. Genet. 1, 327-341, 1982; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 422-426, 1982; Wigler et al., Cell 14, 725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7, 603, 1981; Graham and van der Eb, Virology 52, 456, 1973, and Neumann et al., EMBO J. 1, 841-845, 1982.

Suitable yeast cells include, e.g., *Saccharomyces* spp. or *Schizosaccharomyces spp.* strains, especially *Saccharomyces cerevisiae* or *Saccharomyces kluyveri* strains. Procedures for transforming yeast cells with heterologous DNA and preparing heterologous polypeptides therefrom are described, e.g., by U. S. Patents 4,599,311, 4,931,373, 4,870,008, 5,37 743 (sic) and 4,845,075, all of which are incorporated in this description by reference. The transformed cells are selected on the basis of a phenotype determinable by means of a selectable marker, generally drug resistance or ability to grow in the absence of a particular nutrient, for example, leucine. For use in yeast, the POT1 vector is preferred, which is described in U. S. Patent 4,931,373. The DNA sequence coding for the GLP-2 peptide may be preceded by a signal sequence, and optionally by a leader sequence, e.g., as described above. Further suitable yeast cells are e.g. strains of *Kluyveromyces*, such as, e.g., *K. lactis, Hansenula,* e.g., *H. polymorpha*, or *Pichia*, e.g., *P. pastoris* (see Gleeson et al., J. Gen. Microbiol. 132, 3459-3465, 1986; U. S. Patent 4,882,279).

Other examples of fungal cells are the cells of filamentous fungi, e.g., *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., especially *A. oryzae, A. nidulans* or *A. niger* strains. For the expression of proteins, the use of *Aspergillus* spp. is described, e.g., in European Patents 272,277 and 230,023, and the transformation of *F. oxysporum* may be carried out, e.g., as described by Malardier et al. (Gene 78, 147-156, 1989).

When a filamentous fungus is employed as the host cell, it is conveniently transformed with the GLP-2-coding DNA construct in such a way that the DNA construct is integrated into the host chromosome, to obtain recombinant host cell. The integration may generally be considered advantageous, since the DNA sequence is much more likely to persist in the cell. Integration of the DNA constructs into the host chromosome can be carried out by conventional methods, for example by homologous or heterologous recombination.

Transformation of the insect cells and production of heterologous polypeptides in the cells may be carried out as described in U. S. Patents 4,745,051, 4,879,236, 5,155,037 and 5,162,222 and European Patent 397,485, all of which are incorporated herein by reference. The insect cell line employed as host is suitably a *Lepidoptera* cell line such as, e.g., *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (see U. S. Patent 5,077,214). The culture conditions may suitably be as described, e.g., in International Patents WO 89/01029 or WO 89/01028, or in the aforementioned references.

The transformed or transfected host cells described above are then cultivated in a suitable nutrient medium under conditions that permit the expression of the GLP-2 peptide, after which the resulting GLP-2 peptide is recovered from the culture.

The nutrient medium used for culturing the cells may be any conventional nutrient medium suitable for culturing the host cells, for example a minimal or complex nutrient medium containing appropriate supplements. Suitable nutrient media are commercially available, or can be produced on the basis of published recipes (e.g., recipes in the catalogs of the American Type Culture Collection). The GLP-2 peptides produced by the cells can be recovered from the nutrient medium by conventional methods, including separation of the host cells from the medium by centrifugation or filtration, precipitation of the protein-like components from the supernatant or filtrate by means of a salt, for example, ammonium sulfate, then purification by various chromatographic procedures, e.g., ion-exchange chromatography, gel-filtration chromatography, affinity chromatography, or the like.

In the pharmaceutical composition according to the invention, the GLP peptide may be formulated by any established process for the formulation of pharmaceutical compositions, for example in the manner described in Remington's Pharmaceutical Sciences (1985). The compositions may be in a form suitable for systemic injection or infusion, and may, as such, be

formulated with a suitable liquid vehicle, such as, e.g., sterile water or an isotonic solution or glucose solution. The compositions may be sterilized by well-known conventional sterilization techniques. The resulting aqueous solutions may be packaged for use, or filtered under sterile conditions and lyophilized, and the lyophilized preparation may be mixed before administration with sterile aqueous solution. The preparation may contain pharmaceutically acceptable auxiliary substances required to establish suitable physiological conditions, such as buffers, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

The pharmaceutical composition of the present invention may be formulated for nasal, dermal, pulmonal or rectal administration. The pharmaceutically acceptable excipient or solvent used in the composition may be any conventional solid excipient. Examples of solid excipients are, e.g.,lactose, white clay, sucrose, talc, gelatin, agar, pectin, gum arabic, magnesium stearate or stearic acid. Similarly, the excipient or solvent may contain any sustained-release substance known in the art, such as, e.g., glyceryl monostearate or glyceryl distearate, alone or mixed with a wax.

It is particularly advantageous to prepare the composition according to the invention in the form of a sustained-release preparation. As such, the composition may be formulated as GLP-2-peptide-containing microcapsules or microparticles, dispersing the peptide in an appropriate pharmaceutically acceptable biodegradable polymer, such as polylactic acid, polyglycolic acid or a lactic acid/glycolic acid copolymer.

For nasal administration the composition may contain GLP-2 peptide dissolved or suspended in a liquid vehicle, particularly an aqueous vehicle for use as an aerosol. The vehicle may contain auxiliary substances such as solubilizing agents, e.g., polyethylene glycol, surface-active agents, absorption-enhancing agents such as lecithin (phosphatidyl choline) or cyclodextrin, or preservatives such as parabens.

In general, the compounds according to the invention are formulated in unit dosage form, which contain per unit dosage 0.5 to 500 mg of the peptide together with a pharmaceutically acceptable vehicle.

The GLP-2 peptide is advantageous for use in appetite suppression or satiety induction, for example in the prevention or treatment of diseases or irregularities associated with impaired appetite regulation. An example of such a disease or irregularity is obesity and type-II diabetes. The GLP-2 dose administered to a patient will depend on the type and severity of the condition to be treated, and is generally about 10 μg per kg of body weight to about 5 mg per kg of body weight.

In the pharmaceutical composition according to the invention the GLP-2 peptide may be combined with another appetite-suppressing or satiety-inducing agent. One such agent is GPL-1, which has been demonstrated to have a certain appetite-suppressing effect (cf. M. D. Turton et al., Nature 379, 69-72, January 4, 1996).

The invention includes the fact that appropriately labeled GLP-2 peptide, e.g., radioactively tagged GLP-2, may be used to identify a GLP-2 receptor in binding studies using tissue(s) expected to express the GLP-2 receptor, such as the hypothalamus tissue. When the receptor is localized by GLP-2 binding, it may be cloned by expression cloning, i.e., so that the tissue in question is used for preparing a cDNA library, the cDNA is cloned into suitable vectors, the vectors are introduced into an appropriate cell in order to effect expression of the cDNA, then the clone expressing the receptor is identified by binding to GLP-2. Subsequently, a cell line which expresses the receptor in a stable manner may be used in a screening assay for GLP-2 agonists (i.e., those compounds acting on the receptor which induce satiety or suppress the appetite) or GLP-2 antagonists (i.e., those compounds which inhibit the effect of GLP-2 on the receptor, and thus can be used for the treatment of cancer-related anorexia or anorexia nervosa).

Below, the invention is illustrated by the following examples, without in any way intending to limit the scope of the invention.

Example 1. Acid ethanolic extraction of tumor tissue

Anorectic tumors were produced in rats in a previously described manner (Madsen, O. D., et al., Endocrinology 133, 2022-2030, 1993). Fifty anorectic 12C3AN (MSL-G-AN) tumors (at –80°C), corresponding to 50.07 g of wet tissue, were homogenized at 4 °C with 700 mL of acid ethanol (96% ethanol / 0.7 M HCl, 3 : 1 v/v). The homogenization was carried out for 5 minutes in a precooled (4°C) 2-liter Waring Commercial Blender at maximum speed. After

homogenization the mixture was stirred at 4 °C for 16 hours. The mixture was centrifuged at 9000 rpm at 4°C for 1 hour. The volume of the supernatant was reduced by vacuum rotation to 20%. During this procedure, in which a major part of the ethanol was eliminated, a small amount of precipitate was formed. This precipitate was removed by centrifuging at 4°C for one hour at 20,000 rpm. The supernatant which still contained some lipid-like material was filtered and charged into a LiChroprep RP-18 (Merck) column (2.5 x 10 cm) at a flow rate of 4 mL/minute, the column being equilibrated with 0.1% TFA at a flow rate of 2 mL/minute. The column was washed with 100 mL of 0.1% TFA at a flow rate of 4 mL/minute. The bound substance was eluted with 400 mL of 0.1% TFA containing 70% (v/v) acetonitrile. The acetonitrile was eliminated by vacuum rotation and the resulting mixture was lyophilized. After lyophilization the material was dissolved in 50 mL of water and the pH was adjusted to 5.3 with 425 μL of 1 N NaOH. After further adjustment of the mixture to pH 6, a precipitate was obtained. Upon returning to pH 5.3 the precipitate was again dissolved. Hence the pH was left at 5.3 and the mixture was lyophilized.

The total yield of the lyophilized material of 50 tumors was 359 mg of dry powder.

Example 2. First purification step: Filtration on Sephadex G-75

The lyophilized material (278 mg) from the acid ethanol extraction, which corresponds to 38 individual tumors, was redissolved in 20 mL of 1 M acetic acid, and charged into a Sephadex G75 column (5 x 50 cm). The column was equilibrated and eluted with 1 M acetic acid at a flow rate of 55 mL/hour, collecting 10-mL fractions. The absorption measured at 280 nm was recorded for each fraction. The gel-filtration chromatogram is shown in Fig. 1. The separate fractions were combined to give the following five main fractions: G1 (fractions 30-39), G2 (40-45), fraction G3 (46-66), G4 (67-91) and G5 (92-118), and after lyophilization these fractions were subjected to bioassay.

Example 3. Second purification step: Preparative HPLC of group G4

The appetite-suppressing activity of some of the gel-filtration groups showed that the activity is in Group G4, and this group was further fractionated by preparative HPLC. The lyophilized G4 material (corresponding to 80 tumors) was redissolved in 15 mL of 0.1% TFA and pumped into a Vydac 214TP1022 C4 column (2.2 x 25 cm) which was equilibrated in 0.1% TFA. The column

was washed with 20 mL of 0.1% TFA and then with 100 mL of MeCN / H_2O /TFA (10.0 : 89.9 : 0.1) (v/v). The material was eluted at 25°C at a low flow rate (4 mL / minute) with a linear gradient formed from MeCN / H_2O / TFA (10 : 79.9 : 0.1) (v/v) and MeCN / H_2O / TFA (65.0 : 34.9 : 0.1) (v/v) for 110 minutes. The UV absorption was monitored at 214 nm and 280 nm. The HPLC chromatogram (recorded at 280 nm) is shown in Fig. 2. Fractions corresponding to ten main groups were produced as shown in Fig. 2. The volume was reduced to about 25% by vacuum rotation, the fractions were lyophilized and analyzed by bioassay.

The appetite-suppressing activity was found in the G4H9 fraction (Example 6); the peptides of this fraction were analyzed by amino-acid sequence analysis and mass-spectrometric analysis (Example 4).

Example 4. Chemical characterization of the peptides present in fraction G4H9

Using an Applied Biosystems Model 477 gas-phase sequencer, an automated Edman degradation amino-acid sequence analysis was carried out essentially by the method described by the manufacturer. The mass-spectrometric analysis was carried out using an ApI III LC/MS/MS system (Sciex, Thornhill, Ont., Canada). The ratio of the triple quadrupole instrument had a mass/charge (m/z) range of 0-2400, and a pneumatically assisted electrospray (also called ion spray) interface (Bruins, A. P. Covey, T. R. and Henion, J. D., Anal. Chem. 59, 2642-2646, 1987, and Covey, T. R., Bonner, R. F., Shushan, B. I. and Henion, J. D., Rapid Commun. Mass Spectrom. 2, 249-256, 1988). The sample was introduced by means of a syringe-type infusion pump (Sage Instruments, Cambridge, MA) via a capillary fused thereto (75 mm inside diameter) at a liquid flow rate set at 0.5 – 1 mL per minute. The m/z scale of the instrument was calibrated by means of the singly charged ammonium adduct ions of poly(propylene glycols) (PPGs) with unit resolution. The accuracy of mass measurements is generally better than 0.02%.

G4H9 fraction:

The amino-acid sequence of the peptide present in a predominant amount in this fraction was found to be as follows:

H A D G S F S D E M N T I L D N L A T R D F I N W L I Q T K I T D

Measured by mass spectrometry, its molecular weight was 3796.

These peptides are identical to rat GLP-2 peptide (1-33). Found in smaller quantities were the following two peptides:

D F P E E V A I A E E L G R R H A D G S F S D E M N T I L D N L A T R D F I N W L I Q T K I T D and

H D E F E R H A E G T F T S D V S S Y L E G Q A A K E F I A W L V K G R.

These peptides are identical to the rat GLP-2 N-terminally extended with spacer peptide 2 and with the rat GLP-1 peptide (1-36 amide), respectively.

Example 5. Testing procedure for measuring appetite suppression in mice

Mice were deprived of normal food for two days, and on the first day of food deprivation they were given free access to 20% sucrose solution. After the second day of the food deprivation period the mice were injected i.p. with 0.5 mL of test-material-containing solution. Thirty minutes after the injection the mice were placed individually in 15 cm^2 boxes (8 boxes in all) which had a stainless steel grid base and a glass drinking tube which projected into the box. The drinking tube was connected with a tank containing 20% sucrose solution, and had, in its interior, an electrode by means of which it could be detected when the mouse established a drinking contact with the solution by measuring the weak (insensible) current passing through the mouse via an electric apparatus connecting the electrode of the drinking tube and the stainless steel grid base. Consumption of the sucrose solution was measured over a ten-minute period by electrically recording the total amount of contact with the sucrose solution during the test period. The extent of appetite suppression achieved with the test material administered was determined by statistically comparing the sucrose consumption of mice treated with control material (excipient) with that of mice treated with test material. The extent of appetite suppression was expressed as a percentage of the response of the control group.

Example 6. Testing of appetite suppression with fractions containing GLP-2

The appetite suppression of mice was tested (cf. Example 5) after treatment with a test substance. The test substance consisted of extracts of anorectic glucagonoma tumor prepared according to Example 3 (gel-filtration fraction G4) or Example 4 (HPLC fraction G4H7), which were dissolved in phosphate-buffered physiological saline. The test solution containing the lyophilized

material originating from the gel-filtration fraction G4 corresponding to 3.3 tumors reduced sucrose consumption by 72%. Of the 10 subfractions of the gel-filtration fraction G4 (cf. Example 4 and Fig. 2), only the fraction containing GLP-2 G4H9 showed a statistically significant appetite suppression, reducing sucrose consumption by 49% when lyophilized material corresponding to 5.3 tumors was administered.

Example 7. Testing of appetite suppression in mice using synthetic GLP-2

Mice were tested for appetite suppression by the method described in Example 5, after being treated with test material consisting of synthetic porcine GPL-2 dissolved in phosphate-buffered saline. The amino-acid sequence of porcine GLP-2 is as follows

H A D G S F S D E M N T V L D N L A T R D F I N W L L H T K I T D .

Intraperitoneal injection of test solution containing 50 micrograms of synthetic porcine GLP-2 caused a 38% reduction of sucrose consumption.

Example 8. Testing method for measuring appetite suppression in mice

The procedure is the same as that of Example 5, with the difference that instead of a 20% sucrose solution we used a solution of baby formula milk (Complain®). The test material was dissolved in a vehicle consisting of phosphate-buffered physiological saline supplemented with 1% albumin. The test substances dissolved in the vehicle were administered either intravenously (IV) in a volume of 100 microliters, or intracerebroventricularly (ICV) in a volume of 10 microliters.

Example 9. Testing of appetite suppression in mice with synthetic GLP-2

Using the method described in Example 8, mice were tested for appetite suppression after treatment with test material consisting of synthetic human GLP-2. Human GLP-2 has the following amino-acid sequence:

H A D G S F S D E M N T I L D N L A A R D F I N W L I Q T K I T D.

IV injection of test solution containing 3 micrograms of synthetic human GLP-2 caused a 24% reduction of milk consumption, whereas ICV injection of 3 micrograms and 10 micrograms of human GLP-2 resulted in a 32% and 35% reduction of milk consumption, respectively.

Claims

1. Use of a pharmaceutical composition containing the amino acid sequence

   X^1 H X^2 D G S F S D E M N T X^3 L D X^4 L A X^5 X^6 D F I N W L X^7 X^8 T K I T D X^9

   where X^1 is NH_2, DFPEEVAIVEELGRR, DFPEEVTIVEELGRR, DFPEEVNIVEELRRR, or fragments thereof,
   X^2 is Ala or Gly,
   X^3 is Ile or Val,
   X^4 is Asn, Ser or His,
   X^5 is Ala or Thr,
   X^6 is Arg or Lys,
   X^7 is Ile or Leu,
   X^8 is Gln or His, and
   X^9 is OH, Lys, Arg, Arg-Lys, Lys-Arg, Arg-Arg or Lys-Lys,
   together with a pharmaceutically acceptable auxiliary substance or vehicle, for appetite suppression or satiety induction.

2. Use of the composition according to Claim 1, wherein X^1 is NH_2.

3. Use of the composition according to Claim 1, wherein X^2 is Ala.

4. Use of the composition according to Claim 1, wherein X^3 is Ile.

5. Use of the composition according to Claim 1, wherein X^4 is Asn.

6. Use of the composition according to Claim 1, wherein X^5 is Ala.

7. Use of the composition according to Claim 1, wherein X^6 is Arg.

8. Use of the composition according to Claim 1, wherein X^7 is Ile.

9. Use of the composition according to Claim 1, wherein X^8 is Gln.

10. Use of the composition according to Claim 1, wherein X^9 is OH.

11. Use of the composition according to Claim 1, wherein the amino acid sequence of the peptide is

    H A D G S F S D E M N T I L D N L A A R D F I N W L I Q T K I T D,

    H A D G S F S D E M N T I L D N L A T R D F I N W L I Q T K I T D or

    H A D G S F S D E M N T V L D N L A T R D F I N W L L H T K I T D.

12. Use of composition according to any one of Claims 1-11 for the prevention or treatment of diseases of irregularities associated with impaired appetite regulation.

13. Use of composition according to any one of Claims 1-11 for the prevention or treatment of obesity or type-II diabetes.

14. Pharmaceutical composition containing a peptide according to any one of Claims 1-11 together with another appetite-suppressing or satiety-inducing agent.

15. Composition according to Claim 14, wherein said other appetite-suppressing or satiety-inducing agent is glucagon-like peptide 1.

16. Method of treatment of diseases or irregularities associated with impaired appetite regulation, characterized in that any of the peptides according to Claims 1-11 is administered to individuals who need such treatment in a sufficient amount for suppression of the appetite or induction of satiety.

17. Method according to Claim 16, characterized in that the disease or irregularity is obesity or type-II diabetes.

18. Method according to Claim 16, characterized in that the amount of peptide is between approximately 10 μg per kg of body weight and 5 mg per kg of body weight.

19. Method of treatment of diseases or irregularities associated with impaired appetite regulation, characterized in administering to an individual who requires such treatment

any of the peptides according to Claim 11 in sufficient amounts for appetite suppression or induction of satiety.

20. Method according to Claim 19, characterized in that the disease or irregularity is obesity or type-II diabetes.

21. Method according to Claim 19, characterized in that the amount of peptide is between approximately 10 μg per kg of body weight and 5 mg per kg of body weight.

22. Method of treatment of diseases or irregularities associated with impaired appetite regulation, characterized in administering to an individual who required such treatment a sufficient amount of an HLPC fraction of a glucagonoma tumor extract prepared by acid ethanol extraction, gel filtration or preparative HPLC, wherein said fraction is shown as fraction G4H9 in Fig. 2 and which contains GLP-2 as one of its main components, or any individual component of said fraction or any two or more components together, in said individual for appetite suppression or induction of satiety.

23 Method of treatment according to Claim 22, characterized in that the disease or irregularity is obesity or type-II diabetes.

24. Use of the peptides according to Claims 1-11 for the preparation of pharmaceutical composition for the prevention or treatment of diseases or irregularities associated with impaired appetite regulation.

[Illegible signature]
Authorized representative
[Illegible ink stamp]

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: ______________________

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.